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SHORT COMMUNICATION

Preliminary study of micronuclei levels in oral exfoliated cells from patients with periodontitis

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Abstract Micronuclei (MN) are abnormal nuclear structures that arise in dividing cells due to chromosomal breakage or chromosome mis-segregation, and their evaluation in oral exfoliated cells may constitute a reliable and noninvasive, cancer biomarker method. Therefore, it is crucial to assess whether different aspects of oral health can induce micronuclear formation. Because chronic periodontal disease is a prevalent inflammatory condition that may lead to reactive oxygen species generation and DNA damage, the aim of this study was to characterize the frequency of micronuclei according to the periodontal status. For this purpose, we analyzed oral exfoliated cells of 30 patients matched by age and sex (15 control patients with healthy periodontum to mild periodontitis and 15 patients with moderate to severe periodontitis). Our results indicated a 2.3-fold increase in MN basal levels in patients with moderate to severe periodontitis compared to the control patients ($P < 0.001$), suggesting that the periodontal status may affect MN reference levels.

Introduction

Micronuclei (MN) are abnormal nuclear structures that arise in dividing cells due to unrepaired or poorly repaired DNA lesions or to irregular chromosome segregation in mitosis. An MN assay is used to demonstrate cytogenetic effects of environmental and occupational exposure to genotoxic agents.[1] Several factors contribute to their formation in cells, including radiation and chemical exposure, alcohol and tobacco consumption, deficiencies of essential nutrients, and harmful metabolic products such as reactive oxygen species (ROS).[1] Age and sex are also reported to affect

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the MN frequency in lymphocytes.[2] Because the oral mucosa provides a barrier to potential carcinogens, an evaluation of MN levels in oral exfoliated cells (OECs) may constitute a reliable, non-invasive, cancer biomarker method.[1] However, there are very few reports addressing the role of oral health in MN levels in these cells, namely individual factors such as tooth decay or periodontal and prosthetic findings.[3] Chronic periodontal disease is a prevalent inflammatory condition among individuals worldwide. Because inflammation may induce ROS generation and DNA damage, a careful evaluation of the MN frequency in patients with different stages of periodontal disease is particularly relevant to standardize the buccal MN assay and potentiate its diagnostic use. The aim of this study was to characterize levels of MN in OECs from patients with different periodontal statuses.

Materials and methods

Sample

A questionnaire was administered to exclude individuals attending the faculty clinic who did not meet the criteria established for this research, following guidelines and exclusion criteria proposed by the Human Micronucleus International Project (HUMN) (http://www.humn.org).[1,4] Accordingly, the 30 individuals selected (who were 50—65 years of age) were nonsmokers, had no history of cancer or chemotherapy/radiotherapy, had sporadic or moderate alcohol consumption (fewer than two glasses of wine or two beers (33 cl)/day), and had no known exposure to genotoxins. Other exclusion factors included a computed tomographic scan or X-rays performed in the 6 months previous to the study, drug consumption, and edentulism.

Ethical considerations

This investigation was approved by the Ethics Committee of the Faculty of Dentistry of the University of Porto. All participants received oral and written information regarding the study purpose and experimental protocols and provided written informed consent before being enrolled in the study.

Periodontal examination

All participants underwent a periodontal examination during which the periodontal attachment loss (AL) was measured (to the nearest 1 mm) by simple probing to identify the cementoenamel junction and to measure the distance to the base of the pocket. The probing depth was defined as the distance from the soft-tissue margin to the tip of the probe. All teeth were examined, and variables were assessed for six sites per tooth: distovestibular/buccal, median-vestibular/midbuccal, mesiovestibular/mesiobuccal, distolingual/palatine, median-lingual/palatine, and mesiolingual/palatine. Participants were divided into two groups, according to the criteria of the Centers for Disease Control and Prevention (CDC) Periodontal Disease Surveillance Project,[5] matched for age and sex. The control group consisted of 15 patients with healthy periodontum to mild periodontitis (with AL of 0—3 mm), and the case group consisted of 15 patients with moderate to severe periodontitis (with AL $\geq$4 mm).

MN cytome assay

The various cell types present in the oral mucosa, including cells with MN, were evaluated in all participants, in a blinded manner, according to the protocol of Thomas et al[6]: briefly, cells were collected in Saccomanno fixative for a fixed-cell analysis using Schiff reagent (Sigma, St. Louis, MO, USA) for nuclear staining and light green (0.2% w/v, Light Green SF Gurr, VWR, Leuven, Belgium) for cytoplasm contrast. Cells were mounted on a glass slide with Eukitt mounting medium (Eukitt, O. Kindler, Freiburg, Germany) and analyzed by optical and fluorescent microscopy (Zeiss Axiovert 200M microscope, Vienna, Austria) using 400$\times$ magnification. In this study, a minimum of 1000 differentiated cells per participant were evaluated.

Statistical analysis

The data obtained in this study were coded and transferred to a database (SPSS 2010 version 18; Chicago, IL, USA). To evaluate the reliability of the measurement method for diagnosing periodontal disease, we used the Wilcoxon test with a significance level of 5%. The Kolmogorov-Smirnov test ($P < 0.05$) showed that the number of cells with MNs/1000 OECs did not follow a normal distribution, and so a nonparametric method (Mann-Whitney) test was applied to compare the cases and control group.

Results

This study included 30 individuals (10 males and 20 females) with an average age of 57.4 years, selected following the exclusion criteria. Intraobserver agreement was observed for the periodontal diagnostics ($P > 0.4$).

When analyzing the cell types in the two groups studied, we observed differences in numbers of basal, binucleated cells and in the group related to DNA damage (MN and nuclear buds (NBUDs). In determining biomarkers for DNA damage, we observed an average 2.3-fold increase in the MN frequency in the group with medium to severe periodontitis. The average MN frequency in the control group (healthy periodontum to mild periodontitis) was 1.33 ± 0.23 MNs/1000 OECs, whereas in the case group (with medium to severe periodontitis), an average of 3.00 ± 0.46 MNs/1000 OECs was obtained. These results were statistically significant ($P < 0.001$). In terms of the MN distribution, in one case from the control group, no MNs were detected per 2000 cells analyzed, and most patients in this group had one or two MNs/1000 OECs. In contrast, in the group with medium to severe periodontitis, several patients presented with high MN scores of up to six MNs/1000 OECs (Figs. 1 and 2). When analyzing the mean frequency of NBUDs, we observed a 2.45-fold increase in the group with medium to severe periodontitis: 0.6 ± 0.16 NBUDs/1000 OECs in the control group versus 1.47 ± 0.24 NBUDs/1000 OECs in the group with medium to severe periodontitis. These results were statistically significant ($P < 0.006$). Furthermore, we

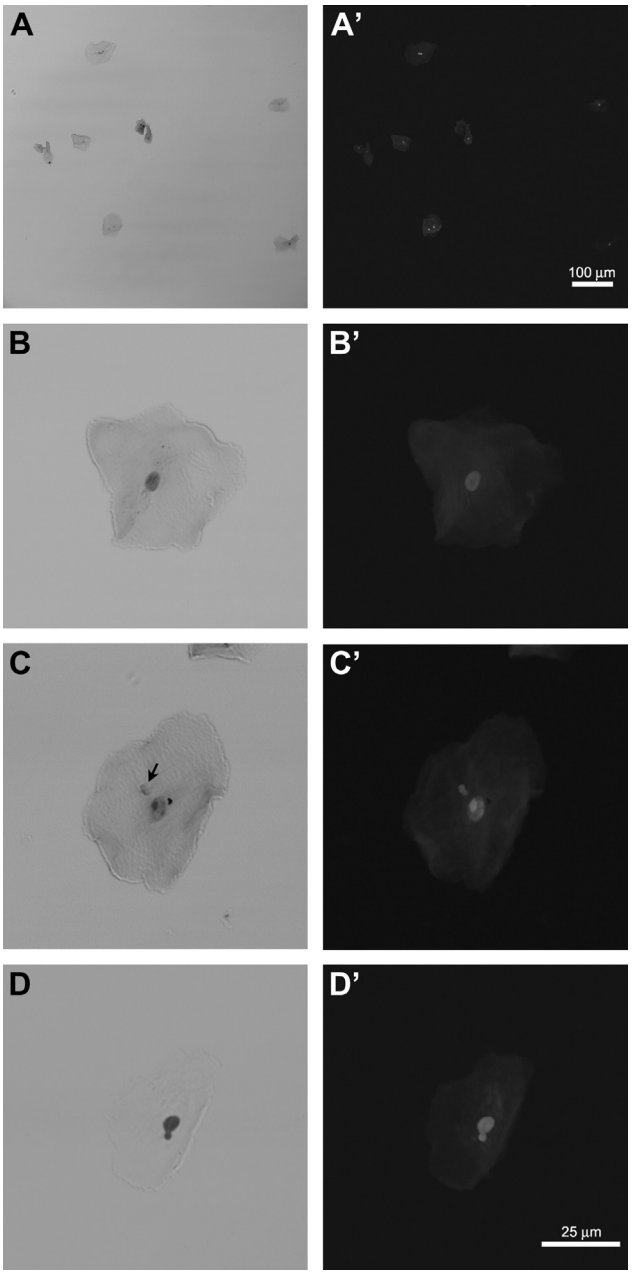

Figure 1 Differentiated cells from the buccal mucosa stained with Schiff reagent and light green. Cells were observed using optical (A—D) and fluorescence microscopy (A′—D′). Differentiated cell (B). Micronucleated cell (C, arrow); these cells are characterized by the presence of both a main nucleus and one or more smaller structures, round in shape, and with a diameter range of approximately one-sixteenth to one-third of the nucleus. Nuclear buds (D); in these cells the main nucleus has a sharp constriction, forming a bud (which is attached) with a diameter range of one-fourth to one-half of the nucleus. Scale bars are 100 μm for panel A/A′ and 25 μm for panels B/B′, C/C′, and D/D′.

observed a higher NBUD score (of up to 3 NBUDs/1000 OECs) in the group with medium to severe periodontitis (Fig. 2). Our results also showed an increase in the mean frequency of binucleated cells in the medium to severe periodontitis

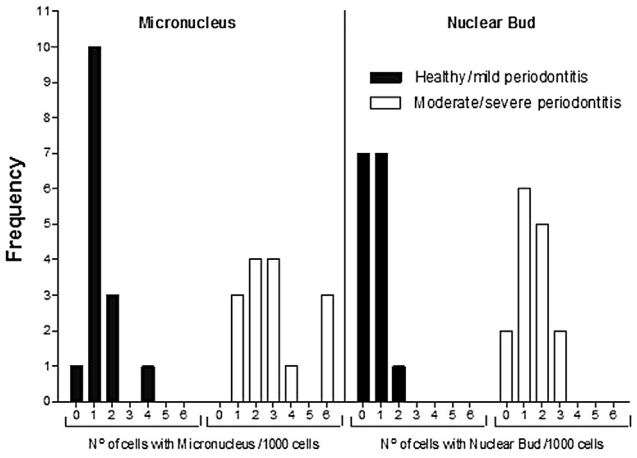

Figure 2 Distribution of micronucleated cells and nuclear bud cells/1000 oral exfoliated cells according to periodontal status.

group of 14.20 cells/1000 OECs, compared to 7.73 cells/1000 OECs in the control group. A similar observation was found in the case of basal cells of 8.73 cells/1000 OECs in the medium to severe periodontitis group compared to 4.87 cells/1000 OECs in the control group (Table 1).

Discussion

The analysis of OECs can be correlated with the proliferative potential (number of basal cells), cell division defects (binucleated cells), cell death (condensed chromatin, karyorrhexis, and pyknotic and karyolitic cells), and DNA damage (MNs and NBUDs).[6] In recent years, OECs were used to show the genotoxic effects of lifestyle factors such as tobacco smoking, betel nut chewing, radiotherapy/chemotherapy treatments, and occupational exposure to radiation and potentially carcinogenic chemicals.[1,6] Although MNs and NBUDs are biomarkers of DNA damage, most of the research focused on MN levels, and several studies showed a statistically significant increase in the frequency of MN in OECs from patients diagnosed with certain types of cancer.[7] Even if this assay has a considerable potential as a biomarker test for cancer, there are many aspects related to confounding factors that need to be addressed. A possible association between the periodontal status and increased MN frequency (compared to restored dental conditions) was suggested in a previous study.[3] However, because other factors such as age, alcohol and tobacco consumption were also reported to affect the MN frequency, we carefully selected participants in this study, and attempted to exclude factors known to interfere with the MN frequency and to narrow the age gap of patients.[2,6] Furthermore, the periodontal status was evaluated using the CDC classification, which is a proposed assessment for population-based surveillance of periodontitis.[5] Therefore, we are confident that our results, even if preliminary, strongly support a correlation between the periodontal status and MN frequency (1.33 ± 0.23 versus 3.00 ± 0.46 for the control group and medium to high periodontitis, respectively, $P < 0.001$). The fact that another cell type,

Table 1 Characterization of the cell types obtained from the participants (per 1000 oral exfoliated cells).

	Basal cell no.	Diff cell no.	MN cell no.	NBUD cell no.	BN cell no.	CC cell no.	KHC cell no.	PYK cell no.	KYL cell no.
Control group (individuals with healthy periodontia or mild periodontitis, $n = 15$)									
Minimum	1.00	602.00	0.00	0.00	1.00	1.00	3.00	1.00	63.00
25% percentile	2.00	701.50	1.00	0.00	4.00	2.50	12.00	3.00	176.50
Median	4.00	747.00	1.00	1.00	6.00	6.00	25.00	6.00	198.00
75% percentile	7.00	771.00	1.50	1.00	13.00	11.50	38.00	13.50	254.50
Maximum	12.00	842.00	4.00	2.00	14.00	42.00	51.00	25.00	335.00
Mean	4.87	734.27	1.33	0.60	7.73	10.07	26.67	8.87	205.60
Standard deviation	3.62	72.18	0.90	0.63	4.88	11.62	16.54	7.79	80.80
Standard error	0.94	18.64	0.23	0.16	1.26	3.00	4.27	2.01	20.86
Lower 95% CI of mean	3.03	697.74	0.88	0.28	5.27	4.19	18.29	4.92	164.71
Upper 95% CI of mean	6.70	770.80	1.79	0.92	10.20	15.94	35.04	12.81	246.49
Case group (individuals with moderate or severe periodontitis, $n = 15$)									
Minimum	2.00	508.00	1.00	0.00	2.00	3.00	11.00	0.00	38.00
Percentile 25%	3.00	606.00	2.00	1.00	6.50	4.00	24.00	6.50	182.00
Median	8.00	662.00	3.00	1.00	16.00	7.00	33.00	13.00	271.00
Percentile 75%	12.00	707.00	3.50	2.00	19.00	11.00	50.00	19.00	331.00
Maximum	24.00	898.00	6.00	3.00	31.00	16.00	83.00	23.00	403.00
Mean	8.73	677.67	3.00	1.47	14.20	7.80	38.33	12.13	236.67
Standard deviation	6.62	111.51	1.77	0.92	8.42	4.44	19.94	7.66	117.37
Standard error	1.71	28.79	0.46	0.24	2.17	1.15	5.15	1.98	30.31
Lower 95% CI of mean	5.38	621.24	2.10	1.00	9.94	5.55	28.24	8.26	177.27
Upper 95% CI of mean	12.08	734.10	3.90	1.93	18.46	10.05	48.42	16.01	296.06
Sig (2—tailed)	$P < 0.081$	$P < 0.056$	$P < 0.002$*	$P < 0.011$**	$P < 0.015$	$P < 0.624$	$P < 0.116$	$P < 0.267$	$P < 0.305$

BN = binucleated; CC = condensed chromatin; CI = confidence interval; Diff = differentiated; KHC = karyorrhectic; KYL = karyolytic; MN = micronucleus; NBUD = nuclear bud; PYK = pyknotic.
*sig (1-tailed): $P < 0.001$.
**sig (1-tailed): $P < 0.006$.

NBUDs, was associated with DNA damage and also showed a 2.45-fold increase in the group with medium to high periodontitis (1.47 $\pm$ 24 NBUDs/1000 OECs compared to 0.6 $\pm$ 0.16 NBUDs/1000 OECs in the control group) further suggests a correlation between the periodontal status and cellular genotoxicity and highlights the importance of standardizing the MN assay in OECs and considering oral health factors. Nevertheless, further studies should be conducted, using a suitable sample, to validate the notion that MN and NBUD levels, and consequently genotoxicity, parallel periodontal severity. Furthermore, as NBUDs may be related to gene duplication or DNA repair and are indicative of DNA damage,[6] a better characterization of NBUD levels in cancer patients and/or individuals exposed to genotoxic chemicals should also be carried out to evaluate their potential as a cancer biomarker.

An important and frequently overlooked aspect of MN research in OECs relates to biomaterials used in endodontic treatment, such as root canal sealers, teeth restoration, and prosthetics. A recent review has adressed several studies which show that metals such as iron, copper, chromium, and cobalt undergo redox cycling reactions and possess the ability to produce reactive oxygen radicals in biologic systems.[8] During orthodontic treatments, corrosion processes may occur that might lead to the release of metals, even though in low concentrations.[9] Biomaterials such as methyl methacrylate, a monomer of acrylic resin that has a wide variety of dental applications, were also shown to induce ROS *in vitro*,[10] and further studies should be conducted to characterize biocompatible dental materials and their effects on patients, namely in terms of the effects of orthodontic appliances, prosthetics, and the use of composite fillings.

Periodontal disease can cause changes in baseline levels of MNs. These preliminary results indicate that a better characterization of oral health factors, which might influence MN levels in OECs, is essential to standardize the buccal MN assay and potentiate its diagnostic value as a biomarker for genotoxicity and cancer.

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